PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter II of the Patent Cooperation Treaty)

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 12467540/EJH/vxk	FOR FURTHER ACTION	See Form PCT/IPEA/416
International application No. PCT/AU2004/000894	International filing date *(day/month/year)* 2 July 2004	Priority date *(day/month/year)* 4 July 2003
International Patent Classification (IPC) or national classification and IPC Int. Cl. [7] C12Q 1/68; C12N 15/12; G01N 33/52		
Applicant GENERA BIOSYSTEMS PTY LTD et al		

1. This report is the international preliminary examination report, established by this International Preliminary Examining Authority under Article 35 and transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 5 sheets, including this cover sheet.
3. This report is also accompanied by ANNEXES, comprising:
   a. [X] *(sent to the applicant and to the International Bureau)* a total of 9 sheets, as follows:
      - [X] sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications authorized by this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions).
      - [ ] sheets which supersede earlier sheets, but which this Authority considers contain an amendment that goes beyond the disclosure in the international application as filed, as indicated in item 4 of Box No. I and the Supplemental Box.

   b. [ ] *(sent to the International Bureau only)* a total of (indicate type and number of electronic carrier(s)) , containing a sequence listing and/or table related thereto, in computer readable form only, as indicated in the Supplemental Box Relating to Sequence Listing (see Section 802 of the Administrative Instructions).

4. This report contains indications relating to the following items:

[X]	Box No. I	Basis of the report
[ ]	Box No. II	Priority
[ ]	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
[ ]	Box No. IV	Lack of unity of invention
[X]	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
[ ]	Box No. VI	Certain documents cited
[ ]	Box No. VII	Certain defects in the international application
[X]	Box No. VIII	Certain observations on the international application

Date of submission of the demand	Date of completion of the report
24 March 2005	19 October 2005
Name and mailing address of the IPEA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized Officer CHRIS LUTON Telephone No. (02) 6283 2256

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/AU2004/000894

Box No. I Basis of the report

1. With regard to the language, this report is based on the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ This report is based on translations from the original language into the following language which is the language of a translation furnished for the purposes of:

☐ international search (under Rules 12.3 and 23.1 (b))

☐ publication of the international application (under Rule 12.4)

☐ international preliminary examination (under Rules 55.2 and/or 55.3)

2. With regard to the elements of the international application, this report is based on (*replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report*):

☐ the international application as originally filed/furnished

☒ the description:

pages 1, 2, 5 – 7, 9 – 11, 13 – 33, 36 – 44 as originally filed/furnished

pages* 3, 4, 8, 12, 34, 35 received by this Authority on 24 March 2005 with the letter of 24 March 2005

pages* received by this Authority on with the letter of

☒ the claims:

pages as originally filed/furnished

pages* as amended (together with any statement) under Article 19

pages* 45 – 47 received by this Authority on 4 October 2005 with the letter of 4 October 2005

pages* received by this Authority on with the letter of

☒ the drawings:

pages 1/7 – 7/7 as originally filed/furnished

pages* received by this Authority on with the letter of

pages* received by this Authority on with the letter of

☐ a sequence listing and/or any related table(s) - see Supplemental Box Relating to Sequence Listing.

3. ☒ The amendments have resulted in the cancellation of:

☐ the description, pages

☒ the claims, page 48.

☐ the drawings, sheets/figs

☐ the sequence listing (*specify*):

☐ any table(s) related to the sequence listing (*specify*):

4. ☐ This report has been established as if (some of) the amendments annexed to this report and listed below had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).

☐ the description, pages

☐ the claims, Nos.

☐ the drawings, sheets/figs

☐ the sequence listing (*specify*):

☐ any table(s) related to the sequence listing (*specify*):

* *If item 4 applies, some or all of those sheets may be marked "superseded."*

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/AU2004/000894

Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims 1-17	YES
	Claims	NO
Inventive step (IS)	Claims 1-17	YES
	Claims	NO
Industrial applicability (IA)	Claims 1-17	YES
	Claims	NO

2. Citations and explanations (Rule 70.7)

The following documents identified in the International Search Report have been considered for the purposes of this report:

D1 – WO 2002/040698

D2 – Cook *et al.*

D3 – Spiro *et al.*

D4 – WO 1997/014028

The invention is seen to reside in a method of detecting aneuploidy in chromosomes using a multiplexing microparticle method comprising producing fluorescently labelled polynucleotide samples that are representative of the abundance of each chromosome in the patient; producing equivalent non-aneuploid polynucleotide standards for each chromosome carrying a different fluorescent label; mixing the labelled sample and standard with an amount of binding agent which comprises a polynucleotide complementary to the sample and standard for each chromosome. The binding agent is immobilised on a microparticle and the microparticles are distinct for each chromosome by size/label/label intensity and wherein the label on the microparticle is distinct from that on the sample and standard. Aneuploidy is then detected as non-equal binding of the sample and standard with the binding agent.

Claims 1- 16 are directed towards detecting aneuploidy in one or more chromosomes of a subject comprising producing fluorescently labelled polynucleotide samples that are representative of the abundance of each chromosome in the patient; producing equivalent non-aneuploid polynucleotide standards for each chromosome carrying a different fluorescent label; mixing the labelled sample and standard with an amount of binding agent which comprises a polynucleotide complementary to the sample and standard for each chromosome. The binding agent is immobilised on a microparticle and the microparticles are distinct for each chromosome by size/label/label intensity and wherein the label on the microparticle is distinct from that on the sample and standard. Aneuploidy is then detected as non-equal binding of the sample and standard with the binding agent.

(continued on extra sheet ...)

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

The term "animal" in claim 5 lacks antecedent basis.

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.

Continuation of: Box V

Claim 17 is directed towards a kit, when used in the method of claim 1, for the simultaneous diagnosis of aneuploidy in one or more chromosomes in a sample comprising fluorescently labelled oligonucleotide primer sets suitable for amplifying chromosome specific sequences; duplicate primer sets comprising a different fluorescent marker to the first marker; a number of binding agents distinct on the basis of microparticle size, reporter and reporter intensity equal to the number of chromosomes in the subject wherein each binding agent is immobilised on a labelled or unlabelled microparticle, said agent comprising a complementary sequence to the predicted amplicon of the primers; instructions for use and where the microparticle label, if present, has a distinct emission spectrum to that of the labelled primers.

NOVELTY (N) and INVENTIVE STEP (IS)

Claims 1-16 are considered novel and inventive as the claimed method is not disclosed or suggested in the citations D1-D4.

D1 discloses the use of multiplex flow cytometry to analyse any soluble factor using multiple parameters such as microparticle size, multiple fluorescent labels and intensities. D1 also discloses that the method is particularly relevant to PCR based assays and that the methods employ competitive hybridisation assays using DNA coupled microspheres and fluorescent DNA probes.

D2 discloses the use of microparticle based flow cytometry to examine cytokine presence in tears.

D3 discloses multiplex bead based identification and quantification of DNA sequences using flow cytometry. The method utilises beads with oligomer capture probes attached to the surface, different fluorophores for multiplexed detection as well as a flow cytometer. The beads capture nucleic acid sequences from a sample and yield a signal intensity for each capture probe which is proportional to the amount of target sequence in the sample. The probes are synthesised using PCR based techniques

D4 discloses the use of multiplex flow cytometry to analyse samples using multiple parameters such as microparticle size, multiple fluorescent labels and intensities. D4 also discloses that the method is particularly relevant to PCR based assays and that the methods employ competitive hybridisation assays using DNA coupled microspheres and fluorescent DNA probes. Applications include genetic variation analysis including mutations and alleles.

In light of the amendment of claim 17 to limit the kit in use to the method of claim 1, claim 17 is novel and involves an inventive step in light of the citations.

Industrial Applicability(IA)

Claims 1-17 meet the requirements of the PCT in regard to Industrial Applicability.

Another type of aneuploidy is triploidy. A triploid individual has three of every chromosome, that is, three haploid sets of chromosomes. A triploid human would have 69 chromosomes (3 haploid sets of 23), and a triploid dog would have 117 chromosomes. Production of triploids seems to be relatively common and can occur by, for example, fertilization by two sperm. However, birth of a live triploid is extraordinarily rare and such individuals are quite abnormal. The rare triploid that survives for more than a few hours after birth is almost certainly a mosaic, having a large proportion of diploid cells.

A chromosome deletion occurs when the chromosome breaks and a piece is lost. This of course involves loss of genetic information and results in what could be considered "partial monosomy" for that chromosome.

A related abnormality is a chromosome inversion. In this case, a break or breaks occur and that fragment of chromosome is inverted and rejoined rather than being lost. Inversions are thus rearrangements that do not involve loss of genetic material and, unless the breakpoints disrupt an important gene, individuals carrying inversions have a normal phenotype.

In a monosomic sample, with 2n-1 chromosomes, one entire chromosome and all its loci are lost. Similarly, in a 2n+1 trisomic sample, one extra chromosome is present in each cell, meaning one specific chromosome is represented three times due to a non-disjunction event, usually in the female gametogenesis. A similar, but more pronounced, situation occurs in the case of a triploid sample in which each chromosome is represented three times instead of twice in each cell.

Pregnancies can be established in infertile women using the technique of *in-vitro* fertilization (IVF). In spite of the high rate of fertilization *in-vitro*, the rate of pregnancy following these procedures is relatively low, ranging from 15% to 25%. Cytogenetic studies of human oocytes fixed after failing to fertilize *in-vitro* display a relatively high incidence of chromosomal abnormalities (aneuploidy). Also, studies of many spontaneous abortions and pre-term embryos show that chromosomal abnormalities may be the main

cause of fetal loss. The frequency of chromosomal abnormality in embryos generated using IVF is much higher than total abnormalities reported for sperm and oocytes.

In the IVF procedure, aneuploidy is the most frequently observed abnormality in the embryos generated. Many reports strongly indicate that chromosomal aneuploidy is the prime cause of fertilization failure in oocytes and implantation failure of embryos. Aneuploidy mainly arises during meiotic non-disjunction: but many environmental factors may also disrupt spindle function and eventually lead to the formation of aneuploid embryos.

Using methods currently known in the art to assess the embryo's gross chromosome makeup, one would perform cytogenetic analyses, such as karyotyping. However, this method is not a practical solution for single cells, and therefore cannot be performed as a pre-implantation screen.

Therefore, there is a need to develop rapid, inexpensive, automatable methods for detecting aneuploidy in an embryo that can be applied in the pre-implantation setting for *in-vitro* fertilization. The present invention provides a method, which has application, *inter alia*, as a rapid, single-tube method for the simultaneous detection of aneuploidy in one, multiple or all chromosomes of a subject.

In particular, this method may increase the success rates of IVF, as embryos with aberrant chromosome numbers (aneuploid) could be screened out by a pre-implantation scan of the embryogenic genetic component.

of the sample and standard. Accordingly, this provides simultaneous measurements for the relative frequency of multiple chromosomes in a sample.

In one aspect, the number of polynucleotides bound to a microparticle derived from a specific chromosome may be from about 1 to about 40,000. In a preferred aspect, the number of polynucleotides bound to the microparticle is from about 1 to about 3,000. In a most preferred aspect, the number of polynucleotides bound to a microparticle is about 2,000.

The method of the present invention has application to the detection of aneuploidy in *any* organism.

In preferred embodiment of the present invention, the subject is a human or other animal embryo generated using *in-vitro* fertilization.

The method of the present invention is able to detect aneuploidy in DNA extracted and/or amplified from a single cell. Therefore, the method of the present application is suitable, *inter alia*, for the detection of aneuploidy in animal embryos generated using *in-vitro* fertilization, prior to implantation of said embryo.

In addition to the detection of chromosome number in an organism, the present invention has application for the detection of non-disjunction events in reproductive cells.

The present invention further provides a kit useful for simultaneously detecting aneuploidy for multiple chromosomes in organism, embryo or reproductive tissue.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for the detection and sorting of microparticles in a mixture of microparticles. The method of the present invention allows for the detection and sorting of many distinct microparticle classes. Detection and sorting is on the basis of microparticle size, the fluorescence spectrum of any attached reporter molecule, the fluorescence intensity of the reporter molecule and discrimination of events based on particle number. These microparticle classes have particular application as binding agents for the detection of aneuploidy in an organism or embryo of the organism. In humans, the detection and sorting of at least 24 classes of microparticles would be sufficient for a single tube method for the simultaneous detection of aneuploidy in all chromosomes, wherein each distinct microparticle class comprises a polynucleotide sequence complementary to, and specific for, a polynucleotide sequence that is unique to a particular human chromosome. Furthermore, using currently available technology, the present method has application for the simultaneous detection of aneuploidy in all chromosomes for an organism that has 216 or fewer pairs of chromosomes. Kits for the simultaneous detection of aneuploidy in one or more human chromosomes are also provided.

Before describing the present invention in detail, it is to be understood that unless otherwise indicated, the subject invention is not limited to specific formulations of agents, manufacturing methods, methodologies, or the like, as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

As used in the subject specification, the singular forms "a", "an" and "the" include plural aspects unless the context clearly dictates otherwise. Thus, for example, reference to "a microparticle" includes a single microparticle, as well as two or more microparticles.

In describing and claiming the present invention, the following terminology is used in accordance with the definitions set forth below.

Current methods in the art for the detection of aneuploidy in embryos include post-implantation screens. Jenderney *et al.* (*Mol. Hum. Reprod.* 6(9): 855-860, 2000) describe the method of using QF-PCR, specific for short tandem repeats on specific chromosomes, on samples of amniotic fluid. It is also possible to assess potential aneuploidy in a fetus from fetal cells in the maternal blood stream, using techniques such as fluorescent *in-situ* hybridization (FISH) (Bianchi *et al.*, *Prenat. Diag.* 22(7): 609-615, 2002). However, as can be seen from the material sampled in these studies, these techniques are only suitable for the detection of aneuploidy in an embryo or fetus post-implantation.

The method of the present invention is able to detect aneuploidy in DNA extracted and/or amplified from a single cell. Therefore, the method of the present application is suitable, *inter alia*, for the detection of aneuploidy in animal embryos generated using *in-vitro* fertilization, prior to implantation of said embryo.

Single cells may be isolated from embryos using standard blastomere biopsy techniques, as will be known to those of skill in the art. Briefly, the blastomere biopsy procedure comprises the following steps:

(i) A 7-cell embryo, on Day 3 after IVF, is ready to be biopsied. It is held in place on a micromanipulator with a holding pipette.

(ii) A zona drilling pipette is used to drill a hole through the shell of the embryo (the zona) using acid Tyrode's.

(iii) The embryo biopsy pipette is then introduced through this opening, and gentle suction is applied to dislodge a single cell (a blastomere) from the embryo.

(iv) The biopsied embryo is then returned to the incubator for further culture. The blastomere can now be screened for aneuploidy according to the method of the present invention.

(v) Based on the analysis of the blastomere, corresponding non-aneuploid embryos are then selected for implantation.

Accordingly, the present invention provides a method for the detection of aneuploidy in an animal embryo generated by *in-vitro* fertilization, prior to implantation of the embryo.

In a preferred embodiment of the present invention, the animal embryo is a human embryo.

In addition to the detection of chromosome number in an organism, the present invention has application for the detection of non-disjunction events in reproductive cells. In this aspect of the present invention, gametes of a said organism, preferably a human, may be tested for missing and/or duplicated chromosomes. The method of this aspect of the present invention would be largely similar to the methods hereinbefore described. Briefly, a nucleic acid representative of a given chromosome in a gamete is labelled with a reporter molecule such as a fluorescent marker, while an equivalent representative polynucleotide from a known non-aneuploid gamete is labelled with a different fluorescent marker. As with the method described for detection of aneuploidy in a somatic or embryogenic cell, the sample and standard polynucleotides are competitively bound to a limiting number of binding agents. A missing chromosome in the sample would be manifest as an increased detection of the standard on the binding agent. Duplication of a chromosome in the sample would be detected as an increased binding of sample to the binding agent. In the case where no non-disjunction events have occurred in the sample, binding of the standard and sample to the binding agent should be approximately equal.

Binding agents contemplated by the present invention comprise a polynucleotide sequence immobilised to a substrate. The polynucleotide sequence of the binding agent comprises a polynucleotide sequence that is complementary to the nucleic acid sequence of the sample and standard, as described *supra*.

By complementary, it is to be understood that an immobilized polynucleotide of the present invention should bind to a chromosome-number representative polynucleotide of

CLAIMS:

1. A method for detecting aneuploidy in one or more chromosomes of a subject simultaneously, said method comprising:

(i) producing fluorescently-labelled polynucleotide samples that are representative of the abundance of each chromosome in said subject;

(ii) further producing equivalent, non-aneuploid polynucleotide standards for each chromosome, labelled with a different fluorescent marker to the sample;

(iii) mixing said sample and standard with a limiting amount of binding agents for each chromosome, wherein said binding agents comprise a polynucleotide that is complementary to the sample and standard for each chromosome immobilized onto a microparticle, and the microparticles for each chromosome are distinct on the basis of size and/or fluorescent label and/or fluorescent label intensity;

wherein the fluorescent label on the microparticle, if present, has a distinct emission spectrum from both the label of the sample and standard; and wherein aneuploidy is detected as non-equal binding of said sample and said standard to said binding agent.

2. The method according to claim 1, wherein the subject is a diploid organism.

3. The method of claim 2, wherein the subject is a mammal.

4. The method according to any one of claims 1 to 3 wherein said subject is a human.

5. The method according to any one of claims 1 to 3 wherein the animal is a livestock animal.

6. The method according to claim 5, wherein the livestock animal is selected from cattle, sheep and horses.

7. The method according to any one of claims 1 to 6, wherein the subject is an embryo.

8. The method of claim 7 wherein said embryo is generated using *in-vitro* fertilization.

9. The method according to claim 7 or 8, wherein said method is suitable for the detection of aneuploidy in said embryo prior to implantation.

10. The method according to claim 9, wherein the DNA sample is isolated, generated or amplified from a blastomere.

11. The method according to any one of claims 1 to 6 wherein the nucleic acid sample and standard are produced from genomic DNA from a somatic cell.

12. The method according to any one of claims 1 to 6 wherein the nucleic acid sample and/or standard are produced from genomic DNA from a reproductive cell or gamete.

13. The method of any one of claims 1 to 12 wherein said binding agent comprises a nucleic acid, with binding specificity for the sample and standard, immobilized on a microparticle.

14. The method according to claim 13 wherein the microparticle is a silica microparticle.

15. The method according to claim 14, wherein the silica microparticle is silanized.

16. The method of any one of claims 1 to 15 wherein the labelled sample and/or standard, and/or relative amounts of labelled sample to standard, are determined using a flow cytometer.

17. A kit when used in accordance with the method of claim 1 for the simultaneous diagnosis of aneuploidy in one or more chromosomes in an organism, embryo or cell, comprising:

(i) fluorescently labelled oligonucleotide primer sets suitable for the amplification of chromosome specific polynucleotide sequences:

(ii) duplicate sets of oligonucleotide primers with identical sequence to the first sets, but comprising a different fluorescent marker with a distinct emission spectrum to the first marker;

(iii) a number of binding agents, distinct on the basis of microparticle size, reporter molecule, or reporter molecule intensity, equal to the number of chromosomes in the subject, wherein each binding agent comprises a polynucleotide sequence complementary to the predicted amplicon of the oligonucleotide primers which is immobilized to a labelled or unlabelled microparticle;

(iv) instructions for the use of said reagents;

wherein the label of the microparticle, if present has a distinct emission spectrum to the label of the both labelled oligonucleotide primers.